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Surveillance for Avian Influenza H_5 Antibodies and Viruses in Commercial Chicken Farms in Kano State, Nigeria

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Abstract: Outbreaks of highly pathogenic Avian Influenza occurred previously for 3 consecutive years, 2006, 2007 and 2008, in Kano State, Nigeria, causing heavy economic losses to farmers and the government. It was against this background that Avian Influenza (AI) surveillance study in commercial poultry farms in the State was conducted. Haemagglutination Inhibition (HI) test was conducted to determine the presence of AI H_5 antibodies in 1,160 sera obtained from flocks in 33 Avian influenza affected (AF) and 25 Non Avian influenza-affected (NAF) farms. To complement the study, 320 cloacal swabs obtained from flocks in farms that were serologically positive for AI H_5 antibodies, were further subjected to Reverse Transcription-Polymerase Chain Reaction (RT-PCR), to determine if the chickens were shedding AI viruses. Of the 1,160 sera tested, 150 (12.9%) were positive for AI H_5 antibodies, with flocks in 16 (27.6%) of the farms being positive. Prevalence rates of 14.1 and 11.4% and mean antibody titres of 5.4±0.2 and 4.6±0.1 $\log_2$ for AI H_5 antibodies were obtained for AF and NAF farms, respectively. The RT-PCR results showed that all the 320 cloacal swabs tested were negative for AI H_5 viruses. The antibodies detected between flocks in the AF and NAF farms might be attributed to vaccination and the titres determined were above the minimum protection level recommended by the OIE. It was recommended that vaccination of chickens against AI should be discouraged because it may interfere with the stamping out policy adopted by Nigeria in the control and eradication of the disease.

Keywords: Antibodies, avian influenza, commercial chickens, Kano, Nigeria, viruses

INTRODUCTION

Nigeria was the first country in Africa to be affected by the Avia Influenza (AI) type A H_5N_1 virus, with Highly Pathogenic Avian Influenza (HPAI) outbreaks initially reported at a commercial farm in Kaduna State in January, 2006 (Adene *et al*., 2006; Sai'du *et al*., 2008). After the first AI outbreak in Nigeria, surveillance efforts in the period between January, 2006 and December, 2007 yielded a total of 299 Nigerian isolates of HPAI H_5N_1 viruses. Mutations at antigenic sites were identified in the haemagglutinin genes of these viruses, the significance of which need to be confirmed by further analyses (Fashina *et al*., 2008). It was reported that the circulating AI H_5N_1 virus during the AI epidemics in Nigeria was a potential candidate for pandemic influenza which may severely affect the human and animal population worldwide especially in the resource-poor countries (Joannis *et al*., 2008). The peak HPAI outbreaks in February 2006 and February 2007 has affected 3,057 farms and farmers; about 1.3 million of the country's 160 million birds were destroyed and the Nigerian government had to pay N900 million (US$5.4 million) in compensation to farmers (FDL, 2008). The disease was reported last in July, 2008, in the States of Kano and Katsina (FDL, 2008). Since that time, efforts to carry out active surveillance for the influenza viruses have been intensified by the national authority. The fact that AI is now endemic in Egypt justifies that researchers in Nigeria should also place AI viruses under constant surveillance. This study was undertaken to screen flocks for AI H5 antibodies and viruses in commercial chicken farms in Kano State, as part of an early warning tool in the prevention of AI outbreaks in Nigeria.

MATERIALS AND METHODS

Areas of the study: Kano State was chosen for this study in view of the fact that some farms in the State had experienced repeated outbreaks of HPAI in 2006, 2007 and 2008. The State is located on Latitude 11° 30' 0 N and Longitude 8° 30' 0 E in North-Western Nigeria, with an area of 42,592.8 km^2. The State is comprised of 44 Local Government Areas (LGAs) and is bounded by

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Katsina, Jigawa, Kaduna and Bauchi States. The State has an estimated human population of 9,383,682 people (2006 census) and an estimated poultry population of 3,852,135 birds comprising 3,528,000 rural and 324,135 commercial poultry as at 2003 (Adene and Oguntade, 2006).

Sample size and sampling technique: Based on the assumption of a scenario that 50% of commercial chicken farms may have AI problem, 64 farms were selected by simple random sampling from a list of 128 registered chicken farms obtained from the Desk Office of Avian Influenza Control Project (AICP), Kano State. However, 6 farms declined for the study. Thus, 58 farms comprising 33 AF and 25 NAF farms in 46 villages of 12 LGAs of the State were selected for the study. A total of 1,160 samples were collected from chickens (selected at random) in the selected farms (20 samples per farm irrespective of flock size).

Sample collection: Two millilitres (mL) of blood were collected from the chickens through the brachial vein using 21 gauge needles and 5 mL syringes. The blood was allowed to clot at room temperature. Sera were separated by centrifugation at about 447.2 xg for 5 min. And the sera were stored in the refrigerator at -4°C until used.

Avian influenza H_5N_3 antigen: An AI H_5N_3 antigen was obtained from the Virology Laboratory of St. Jude Childrens Hospital, Memphis, Tennessee, USA, transported in 1% sodium azide and was used for the serological test. The antigen was stored at -60^{o}C until used.

Serological survey for avian influenza H_5 antibodies: One percent Red Blood Cells (RBCs) was first prepared according to the standard protocol described by OIE (2004) and used as indicator. The titre of the antigen was first determined by Haemagglutination test (HA) as previously described (OIE, 2004) and was found to be 10.0 $\log_2$. Antibodies to AI were detected by the Haemagglutination Inhibition (HI) test as previously described (OIE, 2004). The HI titre considered was the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination was assessed by tilting the plates. Only those wells in which the RBCs streamed at the same rate as the control wells were considered to show inhibition. The validity of the test was assessed against a negative control serum, which gave a titre >4 $\log_2$ and a positive control serum for which the titre was <12 $\log_2$.

Molecular survey for avian influenza H_5 viruses: A total of 320 cloacal swabs (20 samples/farm) were collected from flocks in the commercial poultry farms that were serologically positive for the presence of AI H_5 antibodies and further surveyed for the presence of AI H5 viruses using conventional Reverse Transcription-Polymerase Chain Reaction (RT-PCR) according to the method described by Spackman *et al.* (2002) as follows:

Nucleic acid extraction, reverse transcription and Polymerase Chain Reaction (PCR): Nucleic acid extraction was done using QIAmp viral RNA extraction kit (QIAGEN GmbH, Germany) according to manufacturer's recommendation. Viral RNA was eluted in 60 µL elution buffer and reverse transcribed with random primers and Superscript III (Invitrogen, Merelbeke, Belgium). The RNA (template) was used immediately or stored at -80^{o}C. Amplification of resulting cDNA was performed in a 25 µL volume using Chen f and Chen r AI virus (Guan *et al.*, 2002) H5 specific detection primer pairs in the following mixes: RNAase free H_2O (15.9 µL), PCR Buffer (10x) (2.5 µL), $Mgcl_2$ (50 mM) (1 µL), dNTP (10 mM) (0.5 µL), Forward primer (25 µM) (1.25 µL), Reverse primer (25 µM) (1.25 µL). Taq Polymerase (5u/µL), (0.1 µL) and cDNA template (2.5 µL) per sample. PCR thermal programme are as follows: 94°C for 5 min, 40 cycles of 94°C for 30 min, 60°C for 1 min and 72°C for 1 min. Final extention at 72°C for 5 min.

Agarose gel electrophoresis of amplicons: Five µL of amplicons each reaction tube was transferred to a well in a microtitre plate which was mixed with 3 µL gel loading buffer and loaded into tubes of the agarose separately. One Kb plus DNA ladder (Invitrogen®) was used as band maker. Band image documentation and analysis was done using Kodak ID image analysis software (Eastman Kodak Company, 2000) which transmits gel image to a computer monitor.

Data analysis: Data were analyzed using the Statistical Package for Social Sciences (SPSS) software package, version 15 (SPSS Inc., Chicago, IL, USA). Data generated on antibodies were expressed as mean±standard error of the mean (x±S.E.) and reduced into tables. Student's t-test was used to compare the mean antibody titres between flocks in AF and NAF farms. Values of $p \leq 0.05$ were considered significant.

RESULTS

This study indicates that chickens in 16 (27.6%) of the 58 farms surveyed were positive for AI H5 antibodies. Of these, 11 (68.8%) were AF farms and 5 (31.3%) were NAF farms (Table 1). An overall prevalence of 12.9% for AI H5 antibodies was

determined from 1,160 sera obtained from 58 farms. A prevalence of 14.1% was obtained for the AF farms,

Table 1: Prevalence and mean avian influenza H_5 antibody titres of serologically positive chickens in commercial farms in Kano State

S/n	Farm code	Farm category	Location	L.G.A	No. positive (%)	Mean antibody titre±S.E. log_2
1	AF5	Sector 1	Zangon Dawanau	Dawakin Tofa	6 (30)	5.5±0.2
2	AF15	Sector 1	Sarauniya	Dawakin Tofa	7 (35)	4.4±0.3
3	NAF11	Sector 1	Kabi	Dawakin Tofa	12 (60)	3.6±0.1
4	AF36	Sector 1	Kankare	Kumbotso	10 (50)	6.2±0.4
5	AF3	Sector 2	Danbare	Kumbotso	9 (45)	4.8±0.1
6	AF2	Sector 2	Danbare	Kumbotso	11 (55)	5.0±0.1
7	AF18	Sector 2	Zaria rd	Kumbotso	4 (20)	7.2±0.2
8	AF8	Sector 2	Danbare	Kumbotso	6 (30)	4.2±0.2
9	AF31	Sector 1	Nurul Haiwanat	Kumbotso	13 (65)	6.2±0.1
10	AF45	Sector 3	Mariri	Kumbotso	9 (45)	3.8±0.4
11	NAF22	Sector 2	Sallare	Kumbotso	14 (70)	5.6±0.5
12	CO14	Sector 2	Bechi	Kumbotso	4 (20)	2.8±0.3
13	AF26	Sector 2	Jirma	Kumbotso	10 (50)	4.8±0.5
14	AF17	Sector 1	Badawa	Nasarawa	8 (40)	7.4±0.2
15	NAF41	Sector 2	Korau rd	Nasarawa	11 (55)	5.4±0.2
16	NAF52	Sector 2	Ungogo	Ungogo	16 (80)	5.4±0.1
	Total				150 (12.9)	5.1±0.2

AF: Affected farm; NAF: Non affected farm; n = 20

Table 2: Prevalence and mean avian influenza (H_5) antibody titres of chickens in affected and non affected farms in Kano State

Farm category	No. of farms	Total no. of samples obtained	No. of samples positive	No. with titre ≤4 log_2 (%)	Prevalence (%)	Mean±S.E. (log_2)
NAF	25	500	57	38 (61.4)	11.4	4.6±0.1[a]
AF	33	660	93	82 (88.2)	14.1	5.0±0.2[b]
Total	58	1,160	150	130 (86.7)	12.9	5.1±0.2

AF: Affected farm; NAF: Non affected farm; Student t-test: p = 0.015

Table 3: Prevalence and mean avian influenza H_5 antibody titres of chickens in commercial farms in Kano State based on local government areas

S/n	Local government area	No. of farms with positive result	Total no. of samples obtained	No. of samples positive	Prevalence (%)	Mean±S.E. (log_2)
1	Dawakin Kudu	0	80	0	0	0
2	Dawakin Tofa	3	60	25	41.7	4.5±0.2
3	Fagge	0	20	0	0	0
4	Gezawa	0	80	0	0	0
5	Gwale	0	120	0	0	0
6	Kumbotso	10	420	90	21.4	5.1±0.3
7	Kura	0	20	0	0	0
8	Madobi	0	20	0	0	0
9	Municipal	0	60	0	0	0
10	Nasarawa	2	100	19	19.0	6.4±0.2
11	Tarauni	0	80	0	0	0
12	Ungogo	1	100	16	16.0	5.4±0.1
	Total	16	1,160	150	12.9	5.1±0.2

Table 4: Prevalence and mean avian influenza H_5 antibody titres of chickens in commercial farms in Kano State based on scale of production

S/n	Sector	No. of farms sampled	No. of samples obtained	No. of farms positive (%)	No. of positive samples (%)	Mean±S.E. (log_2)
1	3 (200-5,000 birds)	27	540	1 (1.7)	9 (1.7)	3.8±0.4[a]
2	2 (5,000-20,000 birds)	23	460	9 (15.5)	85 (18.5)	5.0±0.3[b]
3	1 (>20,000 birds)	8	160	6 (10.3)	56 (35.0)	5.6±0.2[c]
	Total	58	1,160	16 (27.6)	150 (12.9)	5.1±0.2

Student t-test: [ab]: p = 0.025; [ac]: p = 0.018; [bc]: p = 0.103

while NAF farms had a prevalence of 11.4% for AI H_5 antibodies (Table 2). There was a significant difference (p = 0.015) in the mean antibody titres of flocks between the AF and NAF farms which were 5.4±0.21 and 4.6±0.17 log_2, respectively (Table 2). Of the 12 LGAs, Dawakin Kudu, Kumbotso, Nasarawa and Ungogo had farms with positive chickens, with prevalences of 41.7, 21.4, 19.0 and 16.0% and mean AI H_5 antibody titres of 4.5±0.2, 5.1±0.3, 6.4±0.2 and 5.4±0.1 log_2, respectively (Table 3). And Based on scale of production with respect to biosecurity defined by the FAO (2004), sectors 1, 2 and 3 farms had prevalences of 35, 18.5 and 1.7%, with mean AI H_5 antibody titres of 5.6±0.2, 5.0±0.3 and 3.8±0.4 log_2, respectively (Table 4).

All the flocks from the 16 commercial poultry farms that were serologically positive for avian influenza H_5 antibodies were negative for avian influenza H_5 viruses. The result indicated that there was no presence of any band corresponding to a base pair of 250 kb which is specific for the gene generated by primer pairs.

DISCUSSION

The overall prevalence of 12.9% of AI H_5 antibodies obtained in this study was lower than the prevalence previously reported (18.1%) in a similar study conducted in apparently healthy flocks in Kaduna State, Nigeria (Durosinlorun *et al*., 2010). Also, in contrast to that study, the prevalence determined in the flocks in Kaduna State was related to the presence of ducks on some of the farms. Even though, a significant difference was observed in the overall means of AI H_5 antibody titres of flocks between AF and NAF farms, the mean titres of the flocks in both categories of farms were within protection level against AI when compared with the minimum protective antibody titre of 4.0 $\log_2$ recommended by OIE (2004).

The finding that chickens in Dawakin Tofa and Kumbotso LGAs had the highest prevalence rate may be explained by the fact that these LGAs recorded the highest number of HPAI cases during the outbreaks that occurred previously in the state, coupled with the fact that these LGAs had the highest concentration of commercial poultry farms sited in close proximity when compared with the other LGAs in the State. The potential risks and major detrimental effects of HPAI in areas with a high density of poultry have earlier been reported (Martin *et al*., 2011). The implication is flocks in Dawakin Tofa and Kumbotso LGAs if exposed might pose serious threats in the spread of AI viruses to other locations. The movement of vehicles and people from farm to farm may create conditions that might facilitate the spread of AI viruses once established (Capua and Alexander, 2004; Cardona, 2007). The finding that flocks in both sectors 1 and 2 farms had significantly higher prevalence rates and means titres for AI H_5 antibodies than the sector 3 farms might be attributed to AI vaccination in the medium and large scale farmers. This could be in an attempt by farmers to protect their flocks from the disease, considering the relatively high level of financial investment involved in the sectors 1 and 2 farms.

The finding that viruses were not detected in this study is similar to the report of previous studies conducted in The United Arab Emirates, where antibodies to AI H_5 were serologically detected in multispecies birds, but no H_5 virus was detected after molecular analysis of the serologically positive samples (Obon *et al*., 2009). Even though, the possibility of natural infection with AI H_5 viruses in these chickens may be considered, the presence of AI H_5 antibodies might be attributed to vaccination against AI, which the farmers were speculated to have been doing as a result of fear, born out of their devastating experiences during the HPAI epidemics that occurred repeatedly in the State. There was evidence that inactivated oil emulsion AI vaccines are being used in commercial chickens in the State. This could have far-reaching implications because some scientists have suggested that vaccinated flocks might pose risks for transmitting AI virus to other flocks (Cardona *et al*., 2006) Although, it was reported that vaccination of chickens against AI with inactivated oil emulsion influenza vaccines is known to prevent AI clinical signs and reduce virus shedding and spread, it is important to note that the available vaccines do not induce immunity in chickens, for a number of reasons, including lack of antigenic match between the vaccine and circulating strain of the virus and insufficient viral antigen in the vaccine (Karunakaran *et al*., 1987; Webster *et al*., 2006). It has also been reported that long-term circulation of the AI virus in a vaccinated population may result in both antigenic and genetic changes in the virus and this has been reported to have occurred in Mexico (Escorcia *et al*., 2008).

Even though the possibility of missing out AI viruses in the cloacal swabs was low, considering the fact that conventional RT-PCR has been shown to detect viruses with titre as low as 3 EID_{50} (50% egg infectious dose) (Joannis *et al*., 2008), the viruses if present might have been detected if techniques with superior sensitivity such as real-time PCR, light cycle real time-PCR and nested PCR were employed (Starick *et al*., 2005; Guan *et al*., 2006).

CONCLUSION AND RECOMMENDATIONS

The results of the study might be an indication that commercial poultry farmers in Kano State are vaccinating their chickens against AI. Similar studies should be conducted in other areas to define the status of AI in Nigeria, in view of the fact that the continued absence of the disease will depend to a large extent on sustained surveillance for the AI viruses.

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